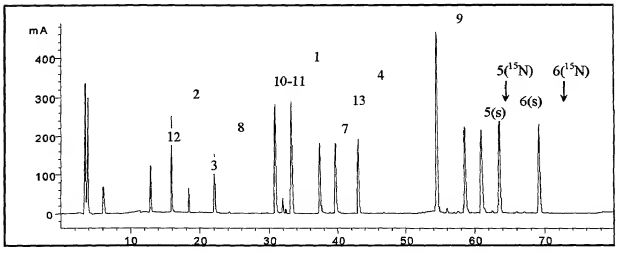

Figure 1. UV traces of a tryptic co-digest of ^{15}N-subtilisin-DAI, indexed (^{15}N), and subtilisin, indexed (s). Peptide numbering refers to Table I.

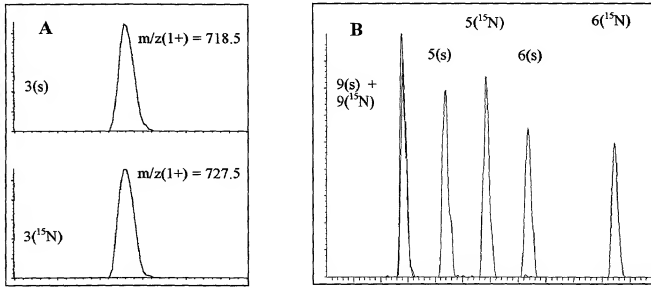

Figure 2. Total ion current chromatogram of selected peptides in Figure 1. (A) Peptide 3 of subtilisin (3 (s), upper panel) and peptide 3 of ^{15}N-subtilisin-DAI (3 (^{15}N), lower panel). (B) TIC of peptides 5, 6, and 9 of the co-digest of ^{15}N-subtilisin-DAI, indexed (^{15}N), and subtilisin, indexed (s). Sequence differences between subtilisin-DAI and subtilisin reside on peptide 5 (N74D) and 6 (S101A, V102I). Amino acid sequence numbering is linear.

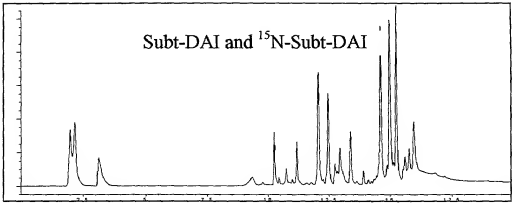

Figure 3. Rapid tryptic digest of subtilin-DAI and ^{15}N-subtilisin-DAI and separation of peptides by RP-HPLC on a 2.0x50 mm C18 column (Jupiter, by Phenomenex). The quantitation by TIC peak area integration of corresponding peaks gave the result expected from enzyme activity assays and active site titrations (see Figures 1 and 2).

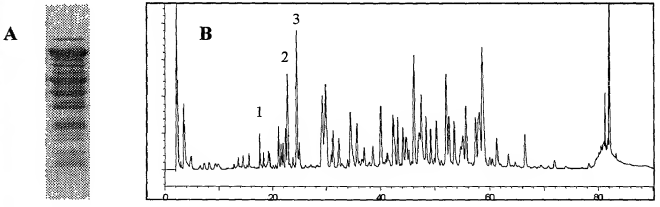

Figure 4. (A) SDS-PAGE of a fermentation broth concentrate of unknown origin. **(B)** This material spiked with a known amount of ^{15}N-labeled purified subtilisin BPN'-Y217L and was digested with trypsin. The peptide mixture was separated by RP-HPLC on a C18 column (2.1 x 150 mm) and the eluate was recorded at 215 nm.

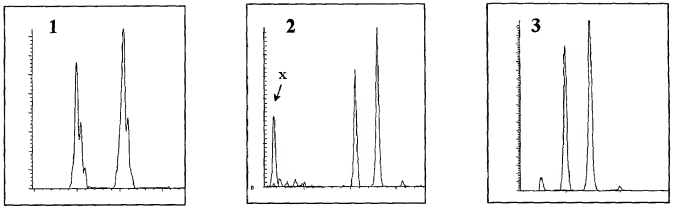

Figure 5. Totoal ion current chromatogram of peptides 1, 2, and 3 from Figure 3. (1) Mass 980.6 (1+), left trace; mass 991.5 (1+), right trace, corresponding to tryptic peptide SSLENTTTK of BPN' and containing 11 nitrogen atoms. (2) Mass 765.6(2+), left trace; mass 775.6 (2+), right trace corresponding to tryptic peptide APALHSQGYTGSNVK of BPN' and containing 20 nitrogen atoms. 'x' is an unrelated peptide. (3) Mass 627.0 (2+), left trace; mass 636.4(2+), right trace corresponding to tryptic peptide HPNWTNTQVR of BPN' and containing 19 nitrogen atoms.

Figure 6.

Table I. Subtilisin-DAI and Subtilisin Tryptic Peptides, Peak Area Ratio from Chromatogram in Figure 1.

Pep #	Sequence	m/z (wt)	m/z (^{15}N)	TIC Peak Area Ratio	UV Peak Area Ratio
1	AQSVPWGISR	1100.58(1+)	1115.53(1+)	1.013	
2	VQAPAAHNR	482.25(2+)	490.23(2+)	1.028	
3	GLTGSGVK	718.40(1+)	727.38(1+)	1.033	
4	VAVLDTGISTHPDLNIR	911.49(2+)	922.45(2+)	0.997	
5	GGASFVPGEPSTQDGNGHGTHVAGTIAALDNSIGVLGVAPSAELYAVK	1531.09(3+)	1549.71(3+)	1.049	
5 (subtilisin)	**N**	1530.77(3+)	-		0.981
6	VLGASGSGAISSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVNSATSR	1642.14	1663.08(3+)	0.979	
6 (subtilisin)	**SV**	1642.80(3+)	-		1.003
7	GVLVVAASGNSGAGSISYPAR	967.51(2+)	979.47(2+)	1.042	
8	YANAMAVGATDQNNNR	855.38(2+)	867.34(2+)	0.971	
9	ASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVK	1600.46(3+)	1619.07(3+)	1.010	
10-11	QK NPSWSNVQIR	729.38(2+)	739.35(2+)	1.044	
12	NHLK	511.29(1+)	519.27(1+)	1.021	
13	NTATSLGSTNLYGSGLVNAEAATR	1185.08(2+)	1200.04(2+)	1.028	
		average peak area ratio:		1.018 ±2.5%	
		average peak area ratio:			0.992 ±1.6%
		average ratio of both methods:		1.005 ±1.3%	
		intended ratio:		1.000	

Figure 7.

Table II. Ratio of concentration and catalytic activity (conversion factor) of 13 variants generated from subtilisin-DAI and expressed in microtiter plates[1]

	Conversion by Peptide Mapping with ^{15}N-Internal Protein Standard		
Variant	EL3.16	OS36.7	EL3.17
Clone 1	0.035	0.015	0.021
Clone 2	0.037	0.014	0.021
Clone 3	0.035	0.015	0.019
Clone 4	0.038	0.014	-
Clone 5	0.038	0.014	-
	Conversion by MBI Titration		
Clone 1	0.036	0.015	0.020

[1] Clones within the three groups, EL3.16, OS36.7, and EL3.17, had the same sequence. Activity was measured by the suc-AAPF-pNA assay (Hsia et al., 1996). The concentration was measured by the peptide mapping method with ^{15}N-labeled subtilisin-DAI as internal standard. The range of concentrations was 2 to 5 □g·ml^{-1}. The conversion factor was verified by an active site titration with a mung bean inhibitor (MBI) solution calibrated on the same plate with a previously calibrated solution of subtilisin-DAI (Hsia et al., 1996).